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Estrogenic Activity in White and Red Wine Extracts

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Red wine is enriched in resveratrol, *trans*-3,5,4′-trihydroxystilbene, a compound in grape skin that inhibits the development of pre-neoplastic lesions in mouse mammary tumor cells in culture and inhibits cancer cell proliferation in vitro. Grapes also contain other bioactive compounds including flavonoids, flavans, and anthocyanins. The estrogenic activities of extracts prepared from one white (Freie Weingärtner Wachau, Grüner Veltliner, Austria) and two red wines (Woodbridge, Cabernet Sauvignon, California; and Lenz Moser Prestige, Blaufränkisch Barrique, Austria) were examined and compared with those induced by estradiol (E_2) and *trans*-resveratrol. First, the estrogenic activity of the wine extracts was evaluated in a yeast estrogen screen (YES) assay, in which yeast express copper-inducible estrogen receptor α (ERα) and an estrogen-response-element (ERE)-driven β-galactosidase reporter. In YES, the white wine extract showed no estrogenic activity. In contrast, both of the red wine extracts showed estrogenic activity equivalent to that of 0.2 nM E_2. Similarly, the white wine extract showed no transcriptional activity with either ERα and ERβ in transiently transfected CHO-K1 cells. In contrast, both red wine extracts stimulated ERE-reporter activity in a concentration-dependent manner that was inhibited by 4-hydroxytamoxifen (4-OHT), indicating that the observed transcriptional activity was ER-mediated. The red wine extracts showed significantly higher ERβ versus ERα agonist activity. Resveratrol showed no agonist activity in YES but activated ERα and ERβ in CHO-K1 cells in a concentration-dependent manner that was inhibited by 4-OHT. This indicates that resveratrol requires mammalian cell components that are absent in yeast for estrogen agonist activity, whereas the estrogenic activity of wine extracts is directly through ERα and does not require mammalian cell factors such as coactivators. The estrogenic activity in red wine found by using YES indicates that estrogenic compounds other than resveratrol are present. Chemical analysis clearly showed that the *trans*-resveratrol content of the red wine extracts was 1 order of magnitude below the detection limit for YES assay.

KEYWORDS: Estrogen receptor; estrogen; resveratrol; phytoestrogen; transcription

INTRODUCTION

The bioflavonoid resveratrol occurs naturally in grapes and has been demonstrated to have both chemopreventive (*1*−*3*) and cardioprotective activities (*4*) in vitro and in animal models. Red wine contains 1−10 mg of resveratrol/L (*5*). Studies on the bioavailability of resveratrol in rats led to the conclusion that even an average consumer of red wine, particularly over the long term, can absorb quantities of resveratrol that correlate with the beneficial health effects of red wine consumption observed in epidemiological studies (*6*−*8*).

The *trans* isomer of resveratrol appears to have greater anticancer and cardioprotective properties than the *cis* isomer (*9*). Resveratrol has been characterized as a phytoestrogen on the basis of its ability to bind to and activate estrogen receptor (ER) (*10*). ER is a nuclear steroid receptor that binds estrogens and regulates the transcription of estrogen-responsive genes by either binding directly to particular DNA sequences called estrogen response elements (EREs) or interacting with other DNA-bound transcription factors, for example, Sp1 (*11*). When activated by an agonist ligand, ERα interacts with coactivators, for example, SRC-1, that either acetylate lysine residues in histones to alter chromatin conformation or interact with components of the RNA polymerase II initiation complex to enhance target gene transcription (*12*).

There are two known ER subtypes: ERα and ERβ (*8*). Although both ER isoforms bind E_2 with comparable affinity,

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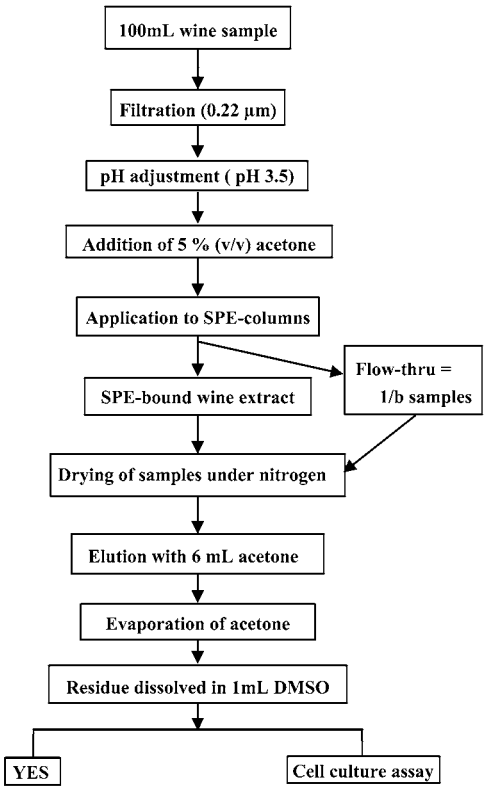

Figure 1. Preparation of wine extracts. This flowchart depicts the general scheme for preparation of the wine extracts used in the experiments.

some phytoestrogens, for example, genistein and coumestrol, show higher affinity for ERβ than for ERα (*5*). We reported that resveratrol binds ERβ and ERα with comparable affinity but that the estrogen agonist activity of resveratrol was greater with ERβ than with ERα, suggesting that those tissues that uniquely express ERβ or that express higher levels of ERβ than ERα may be more sensitive to resveratrol's estrogen agonist activity (*13*). A yeast estrogen screen (YES) has been widely used for assaying the estrogenic activity of pharmacological substances, phytoestrogens, and environmental samples (*14*). YES monitors only pure transactivational activity. If factors other than ERα are required to exert an estrogenic response, YES does not function. Thus, YES provides valuable information when combined with mammalian cell-based transcriptional assays. Difference in response between these two assays provides information on the activation mechanism.

Here we have assayed the estrogenic activity of red and white wine extracts with YES and mammalian-based transcriptional reporter systems and related the resulting data to chemical analysis comprising resveratrol, resveratrol glucoside, anthocyans, and 13 different phenolic compounds characteristic for wines.

MATERIALS AND METHODS

Materials. Three different varieties of wine were tested for estrogenic activity: an Austrian white wine (Grüner Veltliner 1999, Federspiel, Freie Weingärtner Wachau = GV); a California red wine (Cabernet Sauvignon 1997, Woodbridge by Robert Mondavi = WB); and an Austrian red wine (Blaufränkisch Barrique 1999, Lenz Moser Prestige = LM).

Solid Phase Extraction. For removal of solid matter and CO_2 100 mL aliquots of the wine sample were filtered through 0.22 μm filters (Millipore). The pH of the samples was measured and adjusted to pH 3.5 by titration with concentrated H_2SO_4 if necessary. Five milliliters of acetone was added to each 100 mL of wine. **Figure 1** depicts a flow diagram of the sample pretreatment and solid phase extraction procedure.

The samples were then applied to C18 solid phase extraction columns (Bakerbond spe, octadecyl disposable extraction columns, 3 mL; J. T. Baker, Phillipsburg, NJ) by connecting the columns to a water-jet vacuum pump. After sample application, the columns were dried with nitrogen. The adsorbed material was eluted three times with 2 mL of acetone. The acetone was evaporated, and the dry residual matter was dissolved in 1 mL ($^1/_{100}$ of the original sample volume) of DMSO. These samples were subjected to further analysis.

To test the capacity of the solid phase extraction cartridge, the C18 SPE columns were loaded by a 100 mL aliquot of wine. The flow-through of the first column was collected and applied to a fresh second column. Further steps of sample preparation were performed as described above.

Sample Designations. GV is Grüner Veltliner 1999, Federspiel, Freie Weingärtner Wachau (Austrian white wine): GV I, extract of 100 mL of wine sample GV, concentrated 100-fold over one SPE cartridge; GV I/b, flow-through of the first concentration step of wine sample GV concentrated 100-fold over a new SPE cartridge; GV II, extract of another 100 mL aliquot of wine sample GV from the same bottle, concentrated 100-fold over a new SPE column, flow-through discarded; GV III, extract of another 100 mL of wine sample GV from another bottle, concentrated 100-fold, flow-through discarded. LM is Blaufränkisch Barrique 1999, Lenz Moser Prestige (Austrian red wine): LM I, extract of 100 mL of wine sample LM, concentrated 100-fold over one SPE cartridge; LM I/b, flow-through of the first concentration step of wine sample LM concentrated 100-fold over a new SPE cartridge; LM II, extract of another 100 mL aliquot of wine sample LM from the same bottle, concentrated 100-fold over a new SPE column, flow-through discarded; LM III, extract of another 100 mL of wine sample LM from another bottle, concentrated 100-fold, flow-through discarded. WB is Cabernet Sauvignon 1997, Woodbridge by Robert Mondavi (California red wine): WB I, extract of 100 mL of wine sample WB, concentrated 100-fold over one SPE cartridge; WB I/b, flow-through of the first concentration step of wine sample WB concentrated 100-fold over a new SPE cartridge**;** WB II, extract of another 100 mL aliquot of wine sample WB from the same bottle, concentrated 100-fold over a new SPE column, flow-through discarded; WB III, extract of another 100 mL of wine sample WB from another bottle, concentrated 100-fold, flow-through discarded.

Yeast Transactivation Assay. Yeast strain 188R1 (*Saccharomyces cerevisiae*) transformed as a two-plasmid system with an hERα expression plasmid (YEpE12; CUP1 promotor) and a corresponding reporter plasmid (YRpE2) containing two copies of the vitellogenin ERE and the iso-1-cytochrome *c* (CYC 1) in a lacZ fusion vector was used (*15*). The auxotrophy markers were tryptophan (trp) for the expression plasmid and uracil (ura) for the reporter plasmid. Gold medium without trp/ura was used for yeast cultivation. For each experiment, a new overnight culture (30 °C, 180 rpm) was selected and diluted to $OD_{600} = 0.4$ prior to treatment.

Samples of 10, 30, and 50 μL of the concentrated wine extract were added to 5 mL of the diluted yeast culture. To each sample was added the same amount of DMSO. Expression of hERα was induced by the addition of 10 μM $CuSO_4$. DMSO alone was used as a solvent control. A dose−response titration (calibration curve) was performed with 17β-estradiol (E_2) within every experiment. Each treatment was carried out in duplicate within an experiment, and experiments were repeated twice for a total of three separate experiments. After 4 h of incubation at 30 °C, the yeast cells were extracted.

Extraction of Yeast Cells. Cells were collected by centrifugation at 2500 rpm for 5 min. The pellets were resuspended in 1 mL of lacZ buffer (100 mM sodium phosphate buffer, pH 7.0, containing 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol), transferred to test tubes, and recentrifuged (5 min at 10000 rpm, 4 °C). The supernatant was discarded, and 100 μL of lacZ buffer were added to the pellet. Disintegration of the yeast cells was performed by vortexing with glass beads (0.25−0.5 mm, Merck). The samples were vortexed three times for 30 s with a rest of 15 s on ice between vortexes. After centrifugation (10 min at 10000 rpm, 4 °C), β-galactosidase and protein assays were performed.

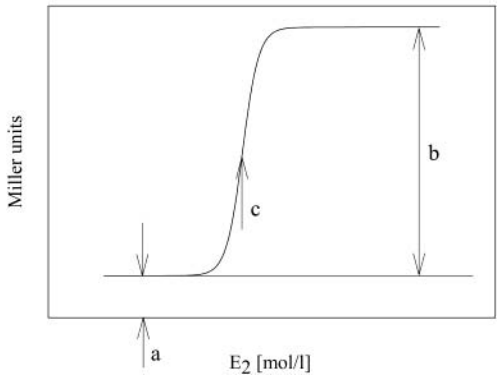

Figure 2. Schematic drawing of E_2 logistic dose response curve in a yeast estrogen screen (YES). By using this calibration curve, the estrogenic activity of the wine extract samples can be expressed in equivalents of E_2.

***β*-Galactosidase and Protein Assays.** Three microliters of the clear supernatant from the samples was transferred into the wells of a 96-well microtiter plate. Two hundred and fifty microliters of a 4 mg/mL solution of the chromogenic substrate *o*-nitrophenyl-*β*-galactopyranoside (ONPG, Sigma) was added, and the plate was incubated at 37 °C for ~14 min until a yellow color had developed. The reaction was terminated by adding 100 μL of 1 M Na_2CO_3. The absorption at 405 nm was measured with an SLT EAR 400 AT plate reader (SLT, Salzburg, Austria).

The amount of total protein was quantified with the Bio-Rad protein assay reagent. A BSA standard curve was created within every test run.

The specific enzyme activity was expressed in Miller units, which take the amount of total protein into account. They are defined as follows, where Δt is the incubation time at 37 °C in minutes:

$$\text{Miller units} = \frac{\text{OD}_{405}}{\mu\text{g of protein mL}^{-1}} * \frac{1}{\Delta t} \times \frac{\text{sample volume protein assay } (\mu\text{L})}{\text{sample volume } \beta\text{-gal assay } (\mu\text{L})} \times 1000$$

Curve Fitting. Data of the transactivation assay were fitted using a logistic dose response model. Calculation was performed with Table Curve 2D software (Jandel Scientific). The function is described as

$$Y = a + \frac{b}{1 + (c/x)^d}$$

Parameter *a* equals the baseline, *b* is the plateau of the curve (ligand efficiency), *c* gives the transition center of the curve (ligand potency), and *d* is related to the transition width (**Figure 2**).

Transient Transfection Experiments. Chinese hamster ovary cells (CHO-K1) were purchased from ATCC (Manasas, VA) and maintained in F12K medium (Gibco BRL, Grand Island, NY) supplemented with heat-treated, 10% newborn calf serum (CS). All other cell culture reagents were purchased from Gibco BRL. For transient transfection, CHO-K1 cells were plated in 24-well plates at 1×10^5 cells/well with Iscove's modified Dulbecco's medium (IMDM, without phenol red) supplemented with 10% charcoal-stripped CS (CSCS). The cells were transfected when 80% confluent with 0.25 μg of reporter construct containing two tandem EREs [2EREc38 (*16*)], 5 ng of pRL-luciferase (*Renilla* luciferase from Promega), 10 ng of pCMV-human ERα or pCMV-rat ER*β* [graciously provided by Dr. B. S. Katzenellenbogen (*17*) and Dr. J.-A. Gustafsson (*18*), respectively]. The transient transfections were performed using Transfast (Promega, Madison, WI) as previously described (*19*−*21*). Cells were treated, in triplicate, 24 h later with ethanol (EtOH or DMSO as vehicle), 0.1, 1, 10, or 100 nM E_2 (Sigma, St. Louis, MO), 100 nM 4-hydroxytamoxifen (4-OHT) (Research Biochemicals International, Natick, MA), 100 nM ICI 182,-780 (Tocris, Ellisville, MO), 10 nM E_2 plus 100 nM 4-OHT, or 10 nM E_2 plus 100 nM ICI 182,780. The wine extracts were used as a 1:1000 dilution in medium, giving the same DMSO dilution as all other treatments. Cells were harvested 24 h later, and luciferase activities were assayed (*19*−*21*). All data for transient transfections were normalized by REN-luc to account for transfection efficiency. Statistical analyses were performed using Student's *t* test or one-way ANOVA followed by Dunnett's post-hoc test using GraphPad Prism.

Cell Proliferation/Viability. MCF-7 human breast cancer cells were purchased from ATCC and maintained in IMEM without phenol red from BioSource International (Camarillo, CA) supplemented with 5% heat-inactivated, charcoal-stripped fetal bovine serum (Atlanta Biologicals, Norcross, GA). Cell proliferation was determined using the cell proliferation kit 1 (MTT) according to the directions provided by the supplier (Boehringer Mannheim, Indianapolis, IN). Briefly, 1.5×10^4 MCF-7 cells were plated per well in 96-well plates. Cells were treated with DMSO or the compound or wine extract indicated in the figure legend for 48 h. Each treatment was performed in quadruplicate within each experiment. The MTT assay was performed according to the manufacturer's instructions, that is, after 48 h. Ten microliters of MTT was added directly to each well. The plates were incubated at 37 °C and 5% CO_2, for 4 h to allow MTT incorporation and cleavage. Then 100 μL of solubilization solution was added overnight at 37 °C and 5% CO_2 (~8 h) to solubilize the MTT crystals. The absorbance of solubilized formazan product was measured at 595 nm directly in each well using a μQUANT plate reader (Biotech Instruments Inc., Winooski, VT).

HPLC Analysis of Resveratrols. The analysis was made from different SPE extracts than YES. A Waters HPLC system consisting of two pumps (type 510, Fa. Waters Associates Inc., Milford, MA) with an automatic sampler (Waters, WISP 712) and a multiwavelength detector (Waters 490 E), and a Waters 810 baseline data acquisition and calculation program was applied. Chromatographical separation was achieved with a LiChrospher RP-C18 column (250 × 4 mm, 5 μm, Fa. Merck, Darmstadt, Germany) and corresponding precolumn (4 × 4 mm, 5 μm). A solvent gradient consisting of 1 mM phosphoric acid (solvent A) and methanol (solvent B) was used. The chromatographic separation was carried out using a three-stage linear gradient: The elution program involved gradient elution from 0% B to 34% B in 90 min held for 3 min, to 50% B in 95 min, held for 10 min, to 80% in 107 min, held for 20 min. An aliquot of 25 μL was injected with one repetition, the flow rate was set at 1.0 mL, and the substances were quantified with UV detection at 310 nm and external calibration. *trans*-Resveratrol and *trans*-resveratrol glucoside (*trans*-piceid) were identified and quantified with standards. *cis*-Resveratrol and *cis*-resveratrol glucoside were produced from the *trans* forms by UV irradiation ($l = 254$ nm) for 24 h. For the quantification of the *cis*-resveratrols a ratio of molar extinction coefficients of *trans*/*cis* = 3.87:1 was used. Sample cleanup and concentration of phenolic substances were achieved by RP-C18 solid phase extraction.

HPLC Analysis of Hydroxycinnamic Acids and Flavan-3-ols. The analysis was made from SPE extracts different from YES. Separation was achieved with an HPLC (type HP 1090 M with HP 9000 diode array detector, Fa. Agilent, Germany). Two analytical columns (HP-ODS Hypersil RP-18, 5 μm, 200 × 2.1 mm; and HP-ODS Hypersil RP-18, 5 μm, 100 × 2.1 mm) and a precolumn (HP-ODS Hypersil RP-18, 5 μm, 20 × 2.1 mm) were connected in series. A linear gradient consisting of (A) 0.5% formic acid, pH 2.3, and (B) methanol was used. Column oven temperature was set at 40 °C, and detection was carried out at 320 nm. Ten microliter wine samples were directly injected after membrane filtration (0.45 μm). Identification and quantification of substances were performed by standards and external calibration. Monomeric anthocyanins were detected with the same HPLC systems as resveratrols; however, the detection wavelength was set at 520 nm.

RESULTS

Estrogenic Activity of Wine Extracts in YES. Three different wine samples, one white wine (GV) and two red wines (LM and WB), were tested for their estrogenic activity on human ERα in a two-plasmid yeast assay (YES). All samples were concentrated 100-fold as described under Materials and Methods

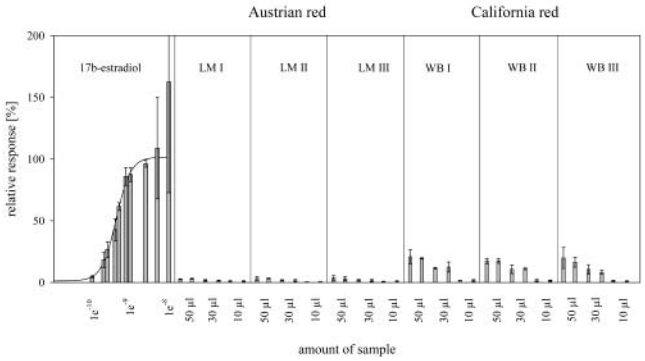

Figure 3. Estrogenic activity of E_2 and concentrated wine samples in YES.

Table 1. Equivalent 17β-Estradiol Activity in Concentrated Wine Samples of Grüner Veltliner (GV) 1999, Federspiel, Freie Weingärtner Wachau, Blaufränkisch Barrique 1999; Lenz Moser (LM) Prestige and Cabernet Sauvignon 1997; and Woodbridge (WB) by Robert Mondavi

wine sample	nM	wine sample	nM	wine sample	nM
GV I	nd[a]	LM I	0.11	WB I	0.29
GV I/b	nd[a]	LM I/b	nd	WB I/b	nd
GV II	nd[a]	LM II	0.12	WB II	0.30
GV III	nd[a]	LM III	0.14	WB III	0.31

[a] Not detectable.

and then assayed for estrogenic activity in the YES (**Figure 3**; **Table 1**). The white wine (GV) had no detectable estrogenic activity in YES (**Table 1**). To confirm that YES is a sensitive method to detect even low estrogenic activity, the detection limit was determined for an E_2 standard curve (**Figure 3**). In fact, this E_2 standard curve was performed each time YES was performed. **Figure 3** shows that the detection limit for estrogenic activity of 17β-estradiol in YES was $\sim 1 \times 10^{-10}$ M.

The samples of the Austrian red wine (LM) showed some ability to transactivate the human ERα in YES (**Figure 3**). An equivalent estrogenic activity was calculated from the E_2 standard curve (**Figure 3**). To evaluate whether the SPE cartridges were overloaded by the applied volume of wine sample (100 mL), we determined the estrogenic activity of sample LM I/b (the flow-through fraction from the SPE column that was subsequently concentrated). No estrogenic activity was detected (data not shown). This means that all substances that are responsible for estrogenic activity bind to the column in the first SPE extraction step and no activity can be detected in the flow-through.

The samples of Woodbridge Cabernet Sauvignon (WB) showed low estrogen agonist activity in YES. The flow-through from the first SPE extraction step (sample WBI/b) did not contain any substances that showed activity in YES. The equivalent E_2 concentrations are shown in **Table 1**. Again, the results of samples WB I, WB II, and WB III are very similar.

Various concentrations of *trans*-resveratrol dissolved in DMSO (1 nM−100 μM) were tested in YES (**Figure 4**). *trans*-Resveratrol showed no estrogenic activity in YES at any of the concentrations tested.

Chemical Analysis. Previous studies by other groups demonstrated that dietary flavonoids exhibit estrogenic activity in breast cancer cells (*22*) and that components in red and white wines inhibit oxidation of isolated human low-density lipoprotein (LDL) in vitro (*23*). Resveratrol was quantified by reversed phase HPLC as free and glucosylated compound. Both isomers were tested (**Table 2**). Total phenol content was analyzed

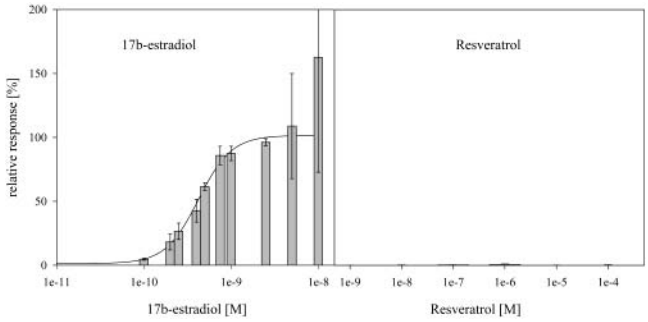

Figure 4. Comparison of E_2 and resveratrol in YES. The assay was performed as described under Materials and Methods and **Figure 3** except that the yeasts were treated with E_2 or resveratrol at the indicated concentrations. Values are the mean of three separate experiments.

Table 2. Contents of Resveratrol, Anthocyans, and Total Phenols in Three Different Wine Samples Determined by HPLC

	GV (white wine)	LM (red wine 1)	WB (red wine 2)
trans-resveratrol glucoside (mg/L)	0.08	1.03	0.17
cis-resveratrol glucoside (mg/L)	0.01	0.86	0.12
trans-resveratrol (mg/L)	0.17	3.06	0.38
cis-resveratrol (mg/L)	0.07	0.90	0.19
monomeric anthocyans (mg/L)	0	84	40
total phenols (mg/L)	50	790	800

Table 3. Contents of Different Phenolic Compounds in Three Different Wine Samples Determined by HPLC

	GV (white wine)	LM (red wine 1)	WB (red wine 2)
caftaric acid (mg/L)	8.1	79.3	13.5
cis-coutaric acid (mg/L)	2.0	4.2	1.9
trans-coutaric acid (mg/L)	1.6	16.2	4.7
fertaric acid (mg/L)	2.2	1.6	0.9
caffeic acid (mg/L)	2.4	5.4	9.5
p-cumaric acid (mg/L)	1600	200	6200
ferulic acid (mg/L)	1.4	0.2	0.2
ethylcaftaric acid (mg/L)	nd[a]	0.1	0.2
ethylcumaric acid (mg/L)	nd	0.1	0.3
gallic acid (mg/L)	2.0	50.0	30.4
tyrosol (mg/L)	13.3	18.6	19.8
catechin (mg/L)	4.9	65.5	33.6
epicatechin (mg/L)	5.1	47.8	13.1

[a] Not detectable.

spectrophotometrically. Both red wine extracts contained resveratrol, anthocyan, and phenol. Phenolic substances were further characterized. Data are shown in **Table 3**.

Estrogenic Activity of Wine Extract in Mammalian Cells. Next we examined the estrogen agonist activity of the wine extracts and resveratrol in CHO-K1 cells. These cells were selected because they express neither ERα nor ERβ (*24*). CHO-K1 cells were transiently transfected with expression vectors for ERα or ERβ and an ERE-luciferase reporter and treated with E_2, resveratrol, or the GV, LM, or WB wine extracts (**Figure 5**). ERα showed higher transcriptional activity in response to E_2 than ERβ, and the activity of each receptor subtype was inhibited by concomitant treatment with 4-OHT, indicating that the E_2-induced luciferase activity was mediated by direct ER binding.

Resveratrol showed concentration-dependent ERα and ERβ agonist activity. ERβ showed greater activation by resveratrol

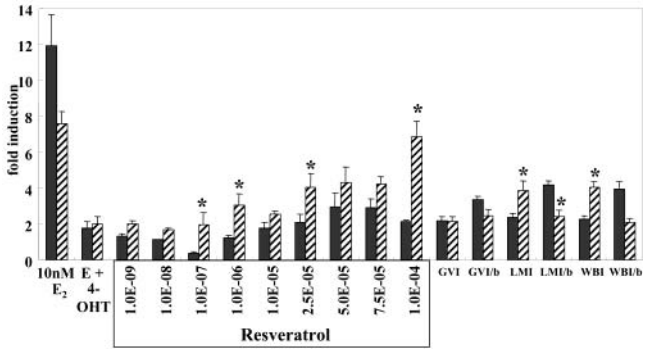

Figure 5. Comparison of resveratrol and wine extract estrogenic activities in transiently transfected CHO-K1 cells. CHO-K1 cells were cotransfected with pGL3-luciferase carrying two tandem copies of the *Xenopus* vitellogenin A2 consensus ERE (2EREc38, sequence under Materials and Methods). The cells were cotransfected with pCMV-ERα or pCMV-ERβ, indicated as different filled boxes, and treated with the indicated concentration of E_2, resveratrol, or a 1:1000 dilution of the indicated wine extract. Cell extracts were assayed for luciferase and *Renilla* luciferase activities as described under Materials and Methods. The fold induction of luciferase activity was normalized for *Renilla* luciferase and is expressed as the ratio of RLU between treatment groups and the vehicle control (which was set to 1). Data are the mean ± SEM from 4–13 different experiments in which each treatment was performed in triplicate within the experiment. An asterisk (∗) indicates values that are significantly different (minimally $p < 0.05$) between ERα and ERβ for the indicated treatment.

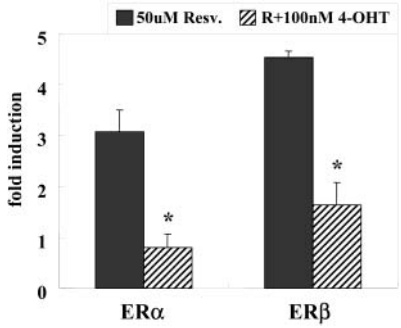

Figure 6. Inhibition of the estrogen agonist activity of resveratrol by 4-OHT. CHO-K1 cells were transfected with ERα or ERβ, indicated by the different filled bars, and treated with 50 μM resveratrol ± 100 nM 4-OHT. The transient transfection assays were performed as described under Materials and Methods and **Figure 5**. Values were normalized to DMSO and are the mean ± SEM of eight separate experiments. An asterisk (∗) indicates values that are significantly different (minimally $p < 0.001$) between resveratrol and resveratrol + 4-OHT.

than ERα. Resveratrol had weaker estrogen agonist activity than E_2. This is similar to our previous findings (*13*) and results comparing resveratrol activity with ERα and ERβ in HepG2 cells (*25*), but contrasts the lack of resveratrol agonist activity with either ERα or ERβ in Ishikawa cells (*26*). The agonist activity of resveratrol was inhibited by 4-OHT, indicating that the agonist activity was mediated by direct resveratrol−ER binding (**Figure 6**).

No significant ERα or ERβ agonist activity was detected with the white wine extracts (GV samples, **Figure 7A**). However, cotreatment of CHO-K1 cells with GV and 4-OHT suppressed activity below basal expression. The SERMS raloxifene, ICI 182,780, and 4-OHT are known to suppress transcriptional activity from reporter genes below basal (vehicle control), although the exact mechanism has not yet been established (*27*).

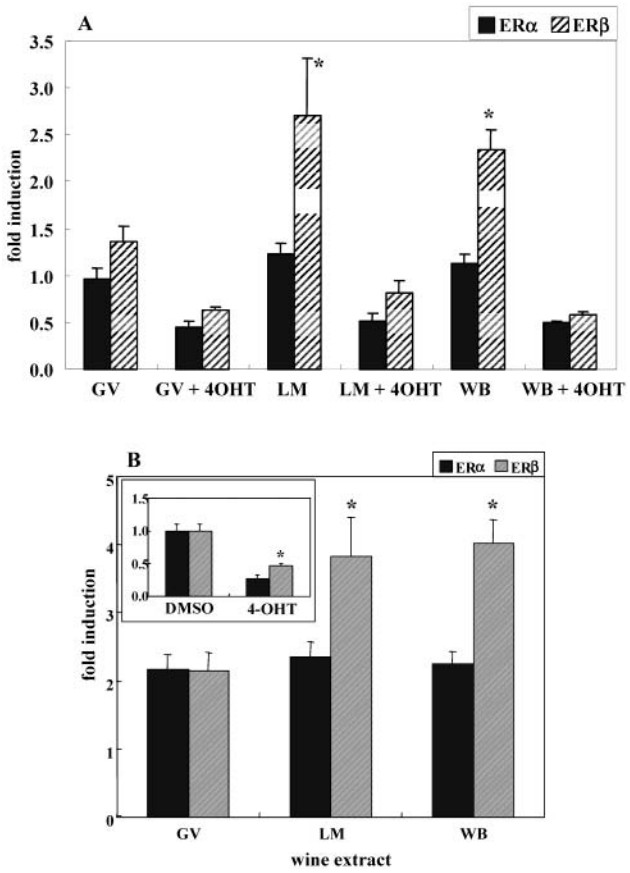

Figure 7. Inhibition of the estrogen agonist activity of wine extracts by 4-OHT. CHO-K1 cells were transfected with ERα or ERβ, indicated by the different filled bars, and treated with a 1:1000 dilution of the indicated wine extract ± 100 nM 4-OHT: (A) values normalized to DMSO; (B) values normalized to 4-OHT. The transient transfection assays were performed as described under Materials and Methods and **Figure 5**. Values are the mean ± SEM of 6–12 separate experiments. An asterisk (∗) indicates values that are significantly different (minimally $p < 0.05$) between ERα and ERβ for the indicated treatment.

To obviate any effect of residual estrogens in the charcoal-stripped serum, the data were normalized to 4-OHT values (**Figure 7B**). These data indicate that GV has similar agonist activity with both ERα and ERβ, that is, a ∼2-fold induction of transcription. This activity is much lower than that detected for 10 nM E_2 (**Figure 5**).

In contrast to the lack of estrogenic activity of white wine, the red wine extracts (LM and WB) showed ERβ-selective estrogen agonist activity (**Figure 7A**). Higher ERβ-selective estrogen agonist activity was detected in LM and WB than in the flow-through samples LM 1/b or WB 1/b (**Figure 5**). The estrogen agonist activity detected in LM and WB was inhibited by 4-OHT, indicating that the activity was mediated by direct binding of some wine extract constituent to ER (**Figure 7A**). To subtract any effect of residual estrogens in the stripped serum used in cell culture from the activity detected in the wine extracts, the values were normalized by the activity detected when the cells were transfected with ERα or ERβ and treated with 4-OHT alone (**Figure 7B**). Both LM and WB showed ∼4-fold induction of luciferase activity with ERβ, a value approximately half that detected with 10 nM E_2.

We also evaluated the estrogen agonist activity of the SPE column flow-through fractions (GV 1/b, LM 1/b,and WB 1/b) (**Figure 8**). None of these extracts showed any estrogenic activity. 4-OHT suppressed ERα and ERβ basal transcriptional

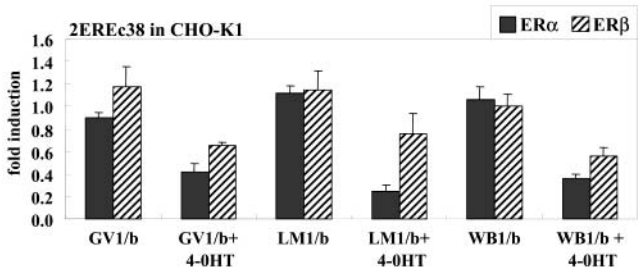

Figure 8. Only the LM "flow-through" fraction contains ERα agonist activity. Data expressed are from the same set of transfections as **Figure 5**, but these data have not been corrected for 4-OHT values. CHO-K1 cells were treated with a 1:1000 dilution of the indicated wine extract flow-through from the SPE column (see diagram) ± 4-OHT. Values are the mean ± SEM of 6–12 separate experiments. An asterisk (cola) indicates a significant difference from control ($p < 0.01$).

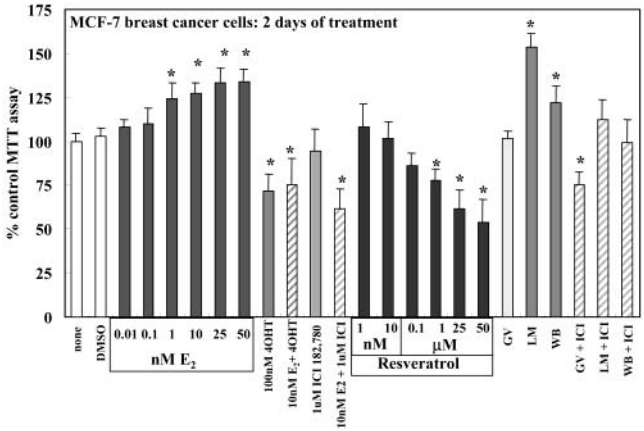

Figure 9. Red wine extracts stimulate the proliferation of MCF-7 breast cancer cells. The effect of the indicated concentrations of E_2, 4-OHT, ICI 182,780, resveratrol, or white (GV) or red (LM and WB) wine extracts on the proliferation of MCF-7 cells was measured by the tetrazolium dye (MTT) assay as described under Materials and Methods. Data are presented as the mean ± SEM of three different experiments in which each treatment was performed in quadruplicate. The absorbance values were converted to percent of DMSO values. An asterisk (∗) indicates values that are statistically different ($p < 0.05$) from control (DMSO) values.

activity below that of basal luciferase expression. The 4-OHT antagonism of the basal activity of ERα was greater than that of ERβ. The exact mechanism for these results is unknown; however, in cells treated with 4-OHT, ERα was shown to recruit corepressors NCoR and SMRT in chromatin immunoprecipitation assays (*28*, *29*). Recruitment of corepressors could suppress basal transcription by keeping chromatin in a condensed state (*30*).

To examine the biological activity of the wine extracts, we determined their impact on MCF-7 breast cancer cell proliferation using the MTT assay (**Figure 9**). MCF-7 cells are "ER positive" and express predominately ERα protein (*31*, *32*). MCF-7 cells treated with E_2 for 2 days showed a concentration-dependent increase in cell proliferation. 4-OHT and ICI 182,-780 inhibited E_2-induced cell proliferation. As reported by us and others (*10*, *13*, *33*−*38*), resveratrol inhibited MCF-7 cell proliferation in a concentration-dependent manner. GV had no effect on cell proliferation, but cotreatment with ICI 182,780 inhibited MCF-7 cell proliferation. Both LM and WB increased MCF-7 cell proliferation, and that increase was blocked by concomitant treatment with ICI 182,780. These results indicate that the two red wine extracts (LM and WB) have estrogenic activity in the MCF-7 breast cancer line that expresses endogenous ERα.

DISCUSSION

The consumption of red wine has been touted as having beneficial effects in terms of preventing cardiovascular disease and cancer (*39*, *40*). Indeed, epidemiological studies have demonstrated that the consumption of wine, particularly red wine, reduces the incidence of mortality and morbidity from coronary heart disease (*41*, *42*). However, other factors including higher social class and educational attainment are correlated with wine consumption, and these factors also correlate with better health (*43*), thus making definitive statements regarding wine consumption and health imprecise. Here we investigated the estrogenic effect of extracts prepared from one white wine and two red wines. Of note, we observed no estrogenic activity in white wine extracts, whereas both red wine extracts showed estrogen agonist activity both in a yeast-based ERα reporter assay, in cells transiently transfected with ERα or ERβ, and in ERα-expressing MCF-7 human breast cancer cells. The red wine extracts both showed higher estrogenic activity with ERβ than with ERα, a result that correlates with the higher affinity of phytoestrogens such as coumestrol and genistein binding to ERβ (*44*).

The key polyphenolic compound implicated in the cardioprotective effects of red wine is resveratrol, *trans*-3,5,4′-trihydroxystilbene. Resveratrol occurs naturally in grapes, with the highest concentration, 50−100 μg of resveratrol/g (*1*), in the grape skin (*45*). Red wine contains 1−10 mg of resveratrol/L (*5*). Chemical analysis showed a content of 0.17 mg/L *trans*-resveratrol for white wine and 3.0 mg/L *trans*-resveratrol for one red wine.

Although resveratrol binds ERα and ERβ with comparable affinity, albeit ∼1 order of magnitude lower than E_2 binding affinity (*13*), here we showed that resveratrol showed higher activation of an ERE-luciferase reporter with ERβ than ERα in transiently transfected CHO-K1 cells. These data are in agreement with our recent report showing that resveratrol-occupied ERβ had higher transcriptional activity than E_2-liganded ERβ at a single palindromic ERE (*13*). This indicates that those tissues that uniquely express ERβ or that express higher levels of ERβ than ERα may be more sensitive to resveratrol's estrogen agonist activity.

One surprising finding was that resveratrol showed no estrogen agonist activity in YES. Clearly, this is not because the YES assay is insensitive because we detected significant agonist activity even at 10^{-10} M E_2. In YES *trans*-resveratrol does not exert estrogenic activity. The highest concentration tested was 10^{-4} M. Ashby and co-workers showed transcriptional activity of resveratrol in a yeast-based ERα assay at concentration ranges $>10^{-4}$ M (*35*). Given that at concentrations $\geq 10^{-4}$ M even testosterone transactivates ERα (our own unpublished results), the specificity of such a response is questionable. From our findings and those of Ashby et al. (*35*), it is clear that YES is not capable of detecting minute quantities of *trans*-resveratrol. At most we found 3 mg/L, which is $\sim 10^{-5}$ M. This is 1 order of magnitude below the detection limit. Even if all conjugated *trans*-resveratrol could be hydrolyzed by yeast-specific enzymes, the concentration of free *trans*-resveratrol would be raised only by one-third, still below the detection limit for YES.

Interestingly, significant transactivation activity was found in red wine by YES. This activity cannot be explained by the resveratrol content. From a comparison of the results in YES

with the mammalian cell culture data, it appears likely that another agonist is present. Importantly, all transactivational activity of the red wines could be antagonized by known antiestrogens, indicating that they must be capable of binding and activating ERα. Recently it has been reported that natural compounds with intrinsic reductive potential may interfere with the MTT assay, leading to false positive results (*46*). We did not observe the characteristic color change reported by Bruggisser et al. (*46*), and we were able to antagonize the estrogenic effect. An unspecific oxidation of the tetrazolium compound cannot be antagonized by tamoxifen.

Assuming that phytoestrogens have an ∼100-fold lower potency than 17β-estradiol and assuming an average relative molecular mass of phytoestrogens of 300 g/mol, ∼10 μg/L of equivalent estrogenic activity could be present in addition to *trans*-resveratrol. This amount could be estimated from the difference between YES and mammalian cell-based assays. All other phenolic compounds must be checked for estrogenic activity, although literature reports do not support the concept that all flavonoids have estrogenic activity (*22*).

Lignans also comprise a substantial fraction of phenolic compounds. Some of these compounds are also weak estrogens. They are considered to have beneficial effects in cardiovascular disease and hormone-dependent breast cancer (*47*). To get a complete overview on the effect of the estrogenicity of wine, these compounds must also be considered.

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